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Toll-like receptor 4 in normal and inflamed lungs and other organs of pig, dog and cattle

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Summary. Bacterial diseases, especially those of the lung caused by Gram-negative bacteria, inflict significant economic loss associated with mortality and morbidity in domestic animals. Toll-like receptor 4 (TLR4) has recently been recognized as a major receptor for cellular interactions with lipopolysaccharides derived from Gram-negative bacteria. However, there are no data on the expression of TLR4 in various organs of domestic animals. We performed immunohistochemistry and immuno-gold electron microscopy to localize TLR4 in lung and seven other organs from normal pig, dog and calf (n=2 each) and in inflamed lungs from calves (n=4) challenged with *Mannheimia hemolytica*. The data show TLR4 in macrophages in lung, small intestine, liver and spleen in all the species and pulmonary intravascular macrophages in calves and pigs. Epithelium in lung, small intestine, cornea and convoluted and straight renal tubules was stained for TLR4. Vascular endothelium of large blood vessels only in lungs and skin was positive, and skeletal muscles were negative for TLR4. In inflamed lungs, airway epithelium showed reduced staining for TLR4 while staining in macrophages remained unaltered. These are the first immunocytochemical data on TLR4 expression in domestic animal species and show similarity in TLR4 staining in macrophages, epithelium and vascular endothelium among dog, pig and cattle.

Key words: TLR4, Immunohistochemistry, Lung inflammation, Immunogold electron microscopy, Pulmonary intravascular macrophages

Introduction

Bacterial diseases such as acute respiratory distress syndrome, bovine pneumonic pasteurellosis and gastro-intestinal disorders cause significant mortality and economic losses in humans and animals (Morsey et al., 1999; Lush and Kvietys, 2000). Bacteria produce endotoxins that induce systemic inflammation in the host. Endotoxins are components of outer cell wall of Gram-negative bacteria and are released during bacterial growth or rapid death. Endotoxins cause fever, leucopenia, tachycardia, hypotension, disseminated intravascular coagulation, and multi-organ failure (Holbrook and Moore, 1994). Similar symptoms can be incited by direct injections of lipopolysaccharides (LPS). Host recognizes and responds to LPS via molecules such as CD14 and Toll-like receptors which are expressed on cells such as monocytes and macrophages (Kang et al., 1990; Labeta et al., 2000; Lee et al., 2000; Takeda et al., 2003).

Toll-like receptors (TLRs) are the mammalian homologues of the *Drosophila* Toll family, which regulates embryo maturation and antimicrobial responses in the adult fly (Takeda et al., 2003). Mammalian TLRs are critical for recognition of conserved pathogen-associated molecular patterns in bacteria and viruses by immune cells (Aderem, 2001). Currently, there are 10 known TLRs including TLR4. TLR4 is expressed on monocytes and macrophages, and is critical for LPS-induced cell signaling through intermediates such as MyD88 eventually leading to nuclear translocation of NF-κB and transcription of proinflammatory genes such as IL-1, TNF-α, IL-6, and IL-8 (Lymboussaki et al., 1998; Aderem, 2001; Flo et al., 2001; Takeda et al., 2003). LPS exposure increases TLR4 expression in human monocytes and macrophages but decreases in mouse peritoneal macrophages and neutrophils (Muzio et al., 2000; Nomura et al., 2000; Kurt-Jones et al., 2002). However, there is evidence that LPS may not affect TLR4 expression in monocytes (Kurt-Jones et al., 2002). TLR4 is also expressed on human dermal endothelial cells and endothelial TLR4 promotes neutrophil recruitment into the lung (Faure et al., 2000; Andonegui et al., 2003). TLR4 has been reported in lung, liver, spleen and kidney of mice and humans (Harju et al., 2001; Zarember and Godowski, 2002). Recently, TLR4 staining was demonstrated in the villi of human placenta and the expression was not

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altered following exposure to LPS (Holmlund et al., 2002). Most of these data were obtained with organ homogenates and, therefore, did not provide information on specific cells expressing TLR4.

Currently, there are no data on the expression of TLR4 in domestic animal species. Because TLR4 regulates innate immune response, we performed light and electron microscopic immunocytochemistry to localize TLR4 in lungs and seven other organs of normal dog, pig and cow. Because lung diseases cause billions of dollars in economic losses to cattle industry, we also examined TLR4 expression in a calf model of acute lung inflammation (Morsey et al., 1999). Now, we report organ and cell specific localization of TLR4 in epithelium, macrophages and endothelium in cattle, pig and dog.

Materials and methods

Tissue and reagents

Normal lung, liver, skin, small intestine, skeletal muscle, spleen, kidney, and eye samples were taken from dog, pig and calf (n=2 each). Inflamed lung tissues were obtained from calves (n=4) infected intra-tracheally with *Mannheimia hemolytica* in other experiments in our laboratory. Bovine serum albumin (BSA) and pepsin were from Sigma, St. Louis, USA. TLR4 antibody and TLR4 peptide were purchased from Santa Cruz Biotechnology, Santa Cruz, USA while rabbit anti-von Willebrand Factor (vWF), horseradish peroxidase (HRP) conjugated anti-goat and anti-rabbit IgGs were from DAKO Diagnostics, Mississauga, Canada. The color development kit for peroxidase and methyl green were purchased from Vector Laboratories Inc., Burlingame, USA.

Light microscopy

The tissues were fixed in 4% paraformaldehyde for 16 hours followed by three washes in phosphate buffered saline (PBS). Tissues were processed for routine paraffin embedding. Five-seven µm sections were prepared from 2 - 3 tissue blocks from each animal, and three sections were placed on poly-L-lysine coated slides. The slides were then incubated at 55°C for 30 minutes to improve the adherence of sections to the slides. Sections were deparaffinized, rehydrated and stained with hematoxylin-eosin to evaluate tissue architecture before proceeding with immunohistochemisty.

Immunohistochemistry

Sections were deparaffinized and rehydrated, followed by incubation with hydrogen peroxide (0.5% in methanol) for 20 minutes to quench endogenous peroxidase. Tissue sections were treated with pepsin (2 mg/ml in 0.01 M HCl) for 45 minutes to unmask antigens and incubated with 1% BSA for 30 minutes to inhibit non-specific binding. Sections were then incubated with anti-TLR4 antibody (4 µg/ml in PBS with 1% BSA, for 60 minutes) followed by appropriate HRP-conjugated secondary antibody (5 µg/ml in PBS with 1% BSA, for 30 minutes) with three washes in PBS in-between. Reaction was visualized using a color development kit for peroxidase and sections were counterstained with methyl green. As a negative control for primary antibody, we used TLR4 antibody after peptide blocking. TLR4 antibody (4 µg) was mixed with 20 µg of blocking peptide in 500 µl of PBS and allowed to react for 2 hours at room temperature. This reaction mixture was mixed with 500 µl of PBS containing 2% BSA to arrive at a concentration of 4 µg/ml of TLR4 in PBS with 1% BSA. Staining with isotype matched goat immunoglobulin (goat IgG; 4 µg/ml) also served as a negative control. Omission of primary antibody and staining with only secondary antibodies served as a negative control for secondary antibodies. Some of the sections were stained without both primary and secondary antibody to determine quenching of endogenous peroxidase. Staining with vWF antibody (1:300) served as a positive control for our immunohistochemistry protocol. Images were captured using a Nikon Coolpix Camera.

Immuno-gold electron microscopy

Lungs from all the species and small intestine from pigs were prepared for immuno-electron microscopy as described previously (Singh et al., 2001). Briefly, tissues fixed in 0.1% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M sodium cacodylate buffer for 3 hours at 4 °C, dehydrated and infiltrated with LR White resin. The tissues were polymerized under ultraviolet light at -8 °C for 3 days. One µm sections were prepared to select areas for ultra-microtomy (100 nm). Sections were stained with anti-TLR4 antibody (1:50) followed by secondary antibody conjugated to 10 nm gold particles (1:100) and examined in an electron microscope at 60kV.

Results

Immunohistochemical controls

We confirmed the specificity of immunohistochemical staining protocols by including a series of controls. First, sections from all the organs including the lung (Fig. 1A) were stained with primary TLR4 antibody after blocking with the TLR4 peptide. This resulted in lack of staining in the sections. Second, substitution of primary antibody with isotype-matched immunoglobulins in the protocol showed no staining in tissue sections (data not shown) Third, sections from all the organs were incubated with only secondary antibody, which also did not show any tissue reactivity (data not shown). Fourth control, staining without primary and secondary antibodies to determine neutralization of

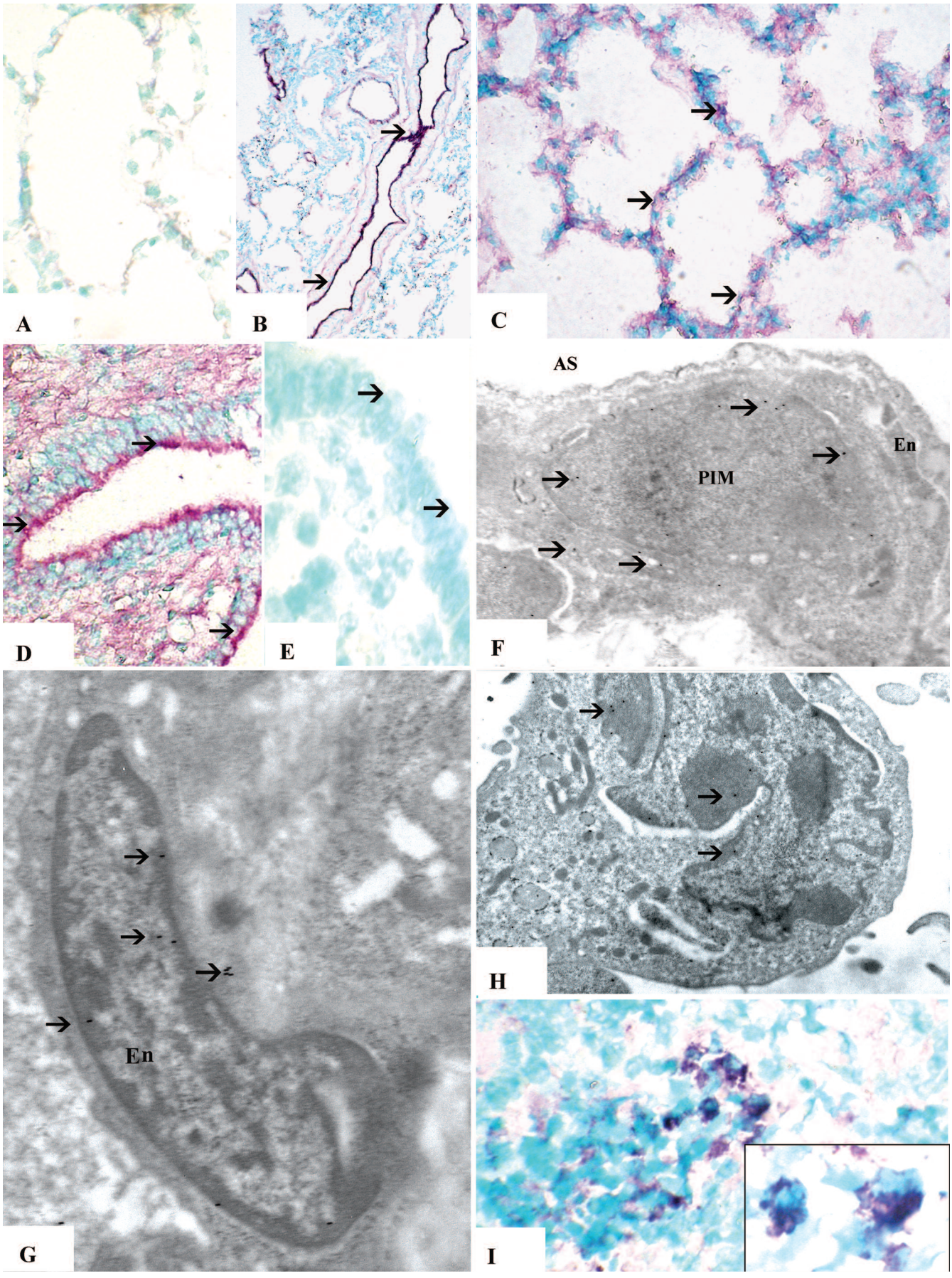
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Fig. 1. Lung immunohistochemistry for TLR4: Staining with TLR4 antibody after peptide blocking served as a negative control and showed no septal staining **(A)** whereas staining with anti-vWF antibody stained only the vascular endothelium (arrows) indicating the specificity of the protocol **(B)**. Alveolar septa (arrows) in a non-inflamed calf lung showed faint staining **(C)**. The staining was intense in bronchiolar epithelial cells (arrows; **D)** of normal calf lung and was not present in the bronchiolar epithelium (arrows) of the inflamed lungs **(E).** Immuno-electron microscopy showed TLR4 labeling (arrows) in pulmonary intravascular macrophages (F; AS: alveolar space; En: Endothelium) large vascular endothelial cells (G; En: Endothelium) and alveolar macrophage **(H)**. In inflamed calf lungs, only the macrophages at the site of inflammation showed TLR4 expression **(I)**. Original magnification: A-C, I, x 40; D, E, I-inset, x 100; F-H, x 10000

endogenous peroxidase, yielded no reaction (data not shown). Lastly, vWF antibody stained the vascular endothelium in all the organs including the lung (Fig. 1B). TLR4 staining in various organs described below was similar across the species and is summarized in Table 1.

Normal lung

Alveolar septa (Fig. 1C) and bronchiolar epithelium (Fig. 1D) showed, respectively, faint and intense reactions for TLR4. Pulmonary monocytes and macrophages were positive for TLR4. Immuno-gold electron microscopy localized TLR4 in pulmonary intravascular macrophages (Fig. 1F), endothelium of large blood vessels (Fig. 1G), alveolar macrophages (Fig. 1H) and bronchiolar epithelium (data not shown).

Inflamed calf lungs

Inflamed lungs from calves infected with *Mannheimia hemolytica* showed classical signs of acute inflammation such as recruitment of inflammatory cells into alveolar spaces (data not shown). Interestingly, TLR4 expression was not present in bronchiolar epithelium (Fig. 1E compared to 1D) and large blood vessels (data not shown) of inflamed lungs compared to the normal lungs. Alveolar macrophages (Fig. 1I) but not neutrophils at the inflammatory foci in the lungs were positive for TLR4.

Small intestine

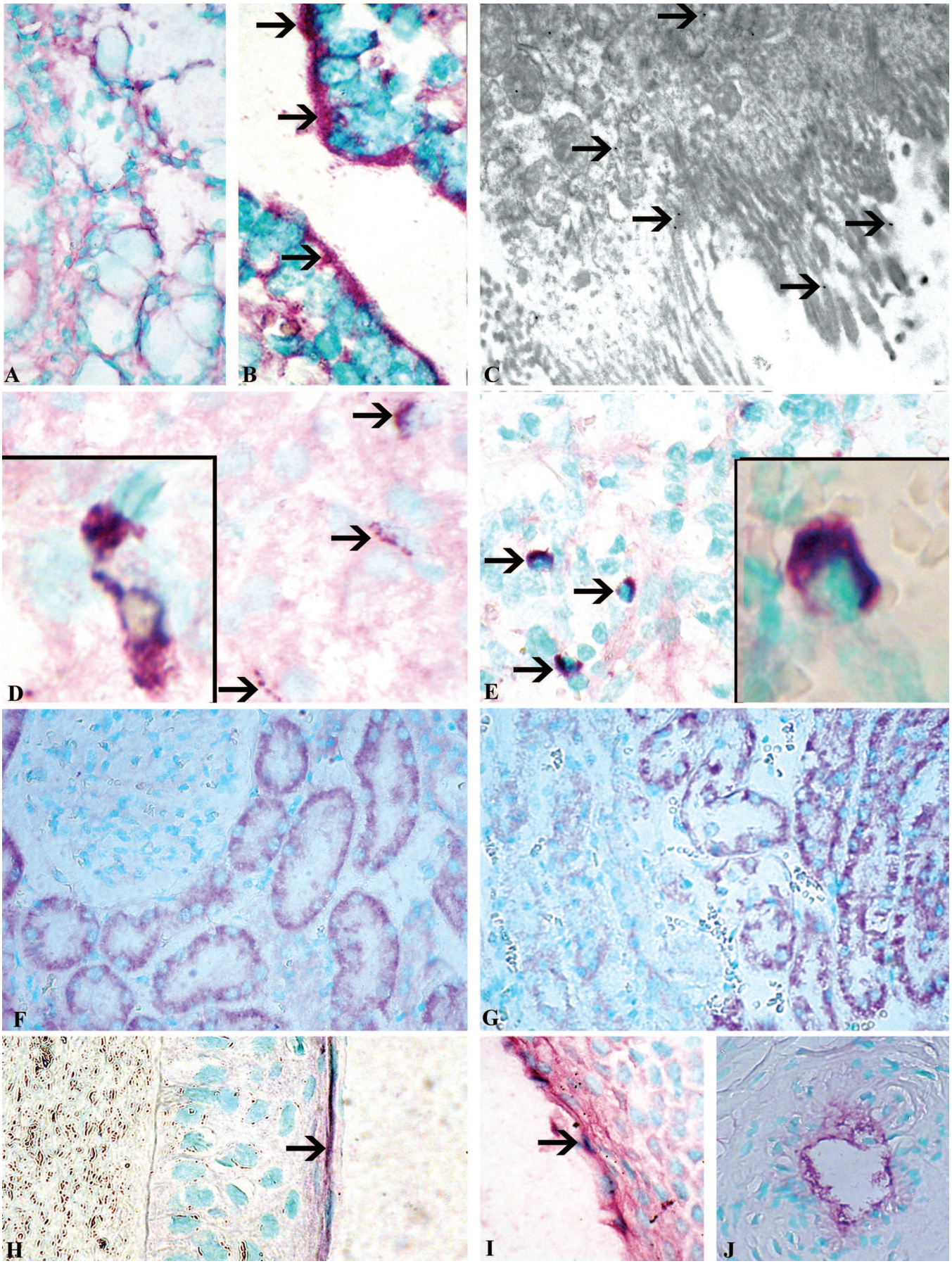
Intense TLR4 staining was observed on the epithelium of small intestine of all the species including the pig (Fig. 2A) and calf (Fig. 2B). Immuno-gold EM confirmed apical and cytosolic staining for TLR4 in small intestinal epithelium of pig (Fig. 2C). Mononuclear cells in the intestine were also positive (data not shown). However, subepithelial layers of small intestine were devoid of TLR4 staining while blood vessels were positive.

Table 1. Immunohistochemical expression of TLR-4 in various organs of cattle, pig and dog.

ORGANS	CATTLE	PIG	DOG
Lung (normal)			
Bronchiolar epithelium	++	++	++
Macrophages	++	++	++
Septa	+	+	+
Lung (inflamed)			
Bronchiolar epithelium	+	+	+
Macrophages	++	++	++
Septa	+	+	+
Small intestine			
Epithelium	++	++	++
Subepithelial layer	-	-	-
Mononuclear cells	++	++	++
Vascular endothelium	++	++	++
Liver			
Kupffer cells	++	++	++
Hepatocytes	-	-	-
Vascular endothelium	-	-	-
Bile duct epithelium	++	++	++
Kidney			
Glomerulus	-	-	-
Convoluted tubules	++	++	++
Collecting ducts	++	++	++
Straight tubules	++	++	++
Vascular endothelium	-	-	-
Skin			
Epidermis	-	-	-
Dermis	-	-	-
Vascular endothelium	-	*	-
Spleen			
Lymphocytes	-	-	-
Macrophages	++	++	++
Vascular endothelium	-	-	-
Muscle	-	-	-
Eyes			
Cornea	++	++	++
Retina and other eye structures	-	-	-

-: No staining; +: Weak staining; ++: Strong staining; *: Occasional blood vessels.

Fig. 2. Immunohistochemistry for TLR4 in intestine, liver, spleen, kidney, eye and skin: Pig **(A)** and calf **(B)** gut epithelia show staining (arrows) for TLR4. Immuno-gold electron microscopy confirmed TLR4 staining (arrows) in the pig gut epithelium **(C)**. Liver (**D** and inset) and spleen (**E** and inset) of dog showed TLR4 reaction in macrophages. The expression in kidney of dog was restricted to convoluted and straight tubules, while renal corpuscle and the vasculature were negative **(F and G)**. Superficial corneal epithelium of dog **(H)** and pig **(I)** was positive (arrows) for TLR4. Occasional blood vessels in skin of pig were positive for TLR4 **(J)**. Original magnification: A, F-J, x 40; B, D-E, x 100; C, x 13000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Liver

TLR4 was localized in Kupffer cells in livers of all the species including dog (Fig. 2D) while hepatocytes and central veins were mostly negative. Occasionally, bile ducts showed luminal staining for TLR4. Liver sections contained minimal background staining.

Spleen

Only macrophages showed reaction for TLR4 in spleens from all the species including the dog (Fig. 2E)

Kidney

Proximal convoluted and the straight tubules of kidneys of all the species including the dog (Fig. 2F,G) were positive for TLR4. Glomerulus, Bowman's capsule and vasculature were negative for TLR4 (Fig. 2F,G).

Eye

TLR4 staining was mostly restricted to corneal epithelium of dog (Fig. 2H), pig (Fig. 2I) and calf (data not shown) while retina and other eye tissues were negative in all the species.

Skin

TLR4 antibody stained occasional blood vessels in the skin especially from the pig (Fig. 2J) while rest of the skin was negative.

Skeletal muscle

Muscle sections from all the species were negative for TLR4 (data not shown).

Discussion

This paper contains the first data on TLR4 localization in lungs and seven other organs of normal pig, dog and cattle. We selected these species because of their veterinary medical significance and for their use as animal models in biomedical research. Pigs are extensively used in studies on organ transplantation and gene therapy (Patel et al., 1999). These species are also physiologically diverse as cattle are polygastric (ruminants) while pig and dog are monogastric (Staub, 1994; Longworth et al., 1996). Cattle are herbivores, dogs are carnivores and pigs are omnivores. Because cattle industry suffers enormous economic loss due to bacterial lung diseases (Morsey et al., 1999), we examined TLR4 expression in inflamed lungs of cattle. Despite the inter-species physiological diversity, our data show similarity in TLR4 staining in macrophages, epithelium and endothelium across the species. Because it is very important to determine the specificity of antibody staining, we included a series of controls including blockade with TLR4 peptide. These controls demonstrated that TLR4 staining seen in tissue sections was true and specific outcome of reaction with TLR4 antibody.

Our data show intense staining for TLR4 in epithelium in lung, cornea, small intestine and convoluted and straight renal tubules. Immuno-electron microscopy detected luminal and cytosolic staining for TLR4 in epithelium of small intestine and airways. Previously, TLR4 protein and mRNA was shown in cultured intestinal epithelial cells and normal monkey ileum (Cario et al., 2000; Hornef et al., 2002; Imaeda et al., 2002). In contrast, another report showed barely detectable TLR4 protein in normal human ileal epithelium; however, it was upregulated in inflammatory bowl disease (Cario and Podolsky, 2000). Therefore, these differences in TLR4 expression in normal intestinal epithelium may be due to inherent inter-species variations.

Because epithelium is the first line of defense against biological or chemical insults, it must contain molecular machinery such as TLR4 for rapid and balanced inflammatory response (Takeda et al., 2003). TLR4 facilitates rapid response including production of inflammatory mediators in airway and other epithelia to inhaled bacteria or endotoxins (Takeda et al., 2003). $TLR4^{-/-}$ mice, intratracheally infected with *Haemophilus influenz*a, show reduced expression of TNF-α and MIP-1 in bronchiolar epithelial cells (Wang et al., 2002). This reduced production of TNF-α and MIP-1 is attributed to lack of TLR4-mediated NF-κB activation, which is central to new cytokine gene transcription (Wang et al., 2002). Interestingly, we noticed reduction or absence of TLR4 staining in airway epithelium in inflamed calf lungs. Although there is evidence of increased TLR4 expression in epithelium of inflamed intestines (Nomura et al., 2000; Hausmann et al., 2002), no such data exist for airway epithelium in inflamed lungs. One possible reason for these differences in TLR4 expression in airway and intestinal epithelial may be the organ-specificity. We also speculate that reduced TLR4 staining in airway epithelium in inflamed lungs may curtail inflammation.

TLR4 was localized in macrophages in normal lungs, liver, spleen and small intestine from all the species. Pulmonary intravascular macrophages of pigs and cattle were also positive for TLR4. TLR4 plays important roles in LPS recognition and activation of human and mice macrophages, and for host defense against bacterial infections (Lymboussaki et al., 1998; Aderem, 2001; Flo et al., 2001; Takeda et al., 2003). This is shown by the data that $TLR4^{-/-}$ mice become resistant to *Pasteurella pneumotropica* infections following reconstitution with $TLR4^{+/+}$ macrophages or bone marrow cells (Hart et al., 2003). TLR4 expression in pulmonary intravascular macrophages may explain their previously reported rapid activation following interactions with blood-borne endotoxins and strong pro-inflammatory potential (Decamp et al., 1992; Staub,

1994; Singh and Atwal, 1997). Furthermore, TLR4 localization in pulmonary intravascular macrophages may partially explain our recent observations that PIM depletion inhibits acute lung inflammation in calves (Singh et al., 2004).

Alveolar macrophages, but not neutrophils, in inflamed lungs showed TLR4 staining similar to the control lungs. Our data agrees with findings of Kurt-Jones and colleagues that monocyte activation does not affect TLR4 expression (Kurt-Jones et al., 2002). However, it differs from previously reported decrease or increase in TLR4 expression in LPS-activated macrophages (Muzio et al., 2000; Nomura et al., 2000). These differences may be partly due to different experimental conditions or species specificity. Neutrophils in inflamed lungs of calves lacked staining for TLR4, which agrees with published data that normally low levels of TLR4 in human neutrophils are further reduced in endotoxemia or upon exposure to LPS (Kurt-Jones et al., 2002; Sabroe et al., 2002; Marsik et al., 2003). Nevertheless, alveolar and intravascular macrophages in inflamed lungs of calves were positive for TLR4, and TLR4 may induce sustained activation in these cells.

We could not detect TLR4 in skeletal muscles from any of the species, which is in contrast to published data on TLR4 expression in skeletal muscle (Lang et al., 2003). An interesting observation was lack of TLR4 expression in vascular endothelium except in large and septal blood vessels in the lung and occasional dermal vessel. Our data on TLR4 staining in lung and dermal blood vessels are in agreement with previous reports (Faure et al., 2000; Andonegui et al., 2002, 2003). TLR4 activates endothelium to induce expression of adhesion molecules such as P-selectin and vascular cell adhesion molecule-1 and recruitment of leukocytes in lung and skin microvessels (Andonegui et al., 2002, 2003). It is generally accepted that leukocyte recruitment in the lung occurs in capillaries but not muscular arteries. However, there is histological data to show leukocyte accumulation around large arteries in inflamed lungs (Pabst and Tschernig, 2002). Therefore, TLR4 in endothelium of large blood vessels, similar to septal capillaries, may provoke perivascular leukocyte recruitment in inflamed lungs. Our data does not address precise reasons for absence of TLR4 in vascular endothelium, in contrast to epithelium, in most of the organs. One possibility may be increased exposure of epithelium compared with endothelium to external environmental challenges. Furthermore, lack of TLR4 in vascular endothelium of liver and other organs and in sensitive layers such as retina may protect them from unwanted inflammation and scar formation.

In summary, our data show expression of TLR4 primarily in epithelium and macrophages of lung and other organs. TLR4 in pulmonary intravascular macrophages may induce their rapid activation following LPS infusion. Inflammation reduced TLR4 staining in airway epithelium but it remained unchanged in alveolar macrophages. These immunocytochemical data require further characterization to understand the precise role of TLR4 in organ inflammation.

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